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Real-Time Identification of Bacteria and *Candida* Species in Positive Blood Culture Broths by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry▽

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Delays in the identification of microorganisms are a barrier to the establishment of adequate empirical antibiotic therapy of bacteremia. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF–MS) allows the identification of microorganisms directly from colonies within minutes. In this study, we have adapted and tested this technology for use with blood culture broths, thus allowing identification in less than 30 min once the blood culture is detected as positive. Our method is based on the selective recovery of bacteria by adding a detergent that solubilizes blood cells but not microbial membranes. Microorganisms are then extracted by centrifugation and analyzed by MALDI-TOF–MS. This strategy was first tested by inoculating various bacterial and fungal species into negative blood culture bottles. We then tested positive patient blood or fluid samples grown in blood culture bottles, and the results obtained by MALDI-TOF–MS were compared with those obtained using conventional strategies. Three hundred twelve spiked bottles and 434 positive cultures from patients were analyzed. Among monomicrobial fluids, MALDI-TOF–MS allowed a reliable identification at the species, group, and genus/family level in 91%, 5%, and 2% of cases, respectively, in 20 min. In only 2% of these samples, MALDI-TOF MS did not yield any result. When blood cultures were multibacterial, identification was improved by using specific databases based on the Gram staining results. MALDI-TOF–MS is currently the fastest technique to accurately identify microorganisms grown in positive blood culture broths.

Blood cultures in liquid medium are the gold standard for the diagnosis of bloodstream infections. Species identification of bacteria that have grown in this biological fluid first requires an overnight subculture on solid agar medium, thus delaying the precise identification of the bacteria by 24 to 48 h. For bacteremic patients, this requirement prevents the rapid prescription of an adequate empirical anti-infective therapy prior to obtaining the results of the antibiotic sensitivity testing. This empirical therapy may be roughly adjusted on the basis of the Gram staining. However, these microscopic results are not accurate enough to reduce the patient's exposure to ineffective antibiotic therapy. In order to reduce the time required for the identification of microorganisms in blood cultures, various methods have been proposed, including identification using automated systems into which fluids from positive blood cultures are directly inoculated, fluorescent *in situ* hybridization (FISH), and PCR followed by sequencing, hybridization, pyrosequencing, or single-stranded conformation polymorphism. All these methods are expensive and require several hours (2, 4, 7–9, 12–15, 17–24, 26, 28, 29).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF–MS) allows rapid identification of bacteria grown on solid media by the identification of species-specific profiles obtained from isolated colonies (3, 5, 25). The adaptation of this technology to the identification of pathogenic microorganisms grown in biological fluids would provide immediate species identification. The advantages of this technique, in addition to its rapidity, are the moderate cost and the ease of implementation. Two recent studies have shown the advantages of MALDI-TOF mass spectrometry applied to positive blood cultures. Correct bacterial identification was obtained in less than 80% of the positive blood cultures and needed several centrifugations, making it difficult to perform the technique each time a blood culture is detected as positive (16, 27). We have developed a strategy where bacteria are released in one step by using a mild detergent that solubilizes blood cells but not bacterial membranes. In this work, we demonstrate the ability of this strategy to identify bacteria from positive blood culture broths in minutes with a good sensitivity.

MATERIALS AND METHODS

Blood and fluid cultures. The preliminary tests used negative blood culture flasks without charcoal (bioMérieux, Marcy l'Etoile, France). They were artificially contaminated with 10^4 cells of commonly isolated pathogens (Table 1) and then placed in the automated blood culture apparatus BacT/Alert (bioMerieux) until detection of positivity. In addition, different pathological fluids from patients were tested, including positive blood cultures (Tables 2 and 3) and different fluids spiked into blood culture flasks (Table 4).

Two aliquots were taken from the blood culture bottle. The first aliquot was taken for MALDI-TOF–MS processing. The second was used for Gram staining, antibiotic susceptibility testing, and appropriate subcultures for microbiological

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TABLE 1. Microbiological identification by MALDI-TOF–MS in blood cultures spiked with different species

Identification obtained from subculture (biochemical or molecular techniques)	No. of samples:					
	Tested	With identification by MALDI-TOF–MS to:				With unacceptable MALDI-TOF–MS profile(s)
		Species	Group[a]	Genus	Family	
Staphylococcus species						
S. aureus	42	42				
S. epidermidis	7	7				
S. haemolyticus	7	6	1 (CNS group)			
S. hominis	3	3				
S. warneri	5	5				
S. lugdunensis	2	2				
Micrococcus luteus	5	3	1 (CNS group)		1	
Streptococcus species						
S. mitis	6		6 (*S. pneumoniae*/*S. mitis* group)			
S. pneumoniae	6		6 (*S. pneumoniae*/*S. mitis* group)			
S. gordonii	4		4 (oral *Streptococcus* group)			
Enterococcus faecalis	14	14				
Gram-negative nonfermenting bacilli						
Pseudomonas aeruginosa	39	38			1	
Stenotrophomonas maltophilia	20	18				2
Acinetobacter baumannii	15	15				
Enterobacteriaceae						
Escherichia coli	20	19				1
Enterobacter cloacae	18	13			5	
Citrobacter freundii	16	14			1	1
Klebsiella oxytoca	17	17				
Klebsiella pneumoniae	20	18	1 (KES group)			1
Proteus mirabilis	18	17		1		
Haemophilus influenzae	2	2				
Anaerobic bacteria						
Clostridium perfringens	2	2				
Fusobacterium necrophorum	3	3				
Bacteroides fragilis	1	1				
Candida albicans	10	10				
Other *Candida* species	10	10				
Total	312	279	19	1	8	5

[a] CNS group, coagulase-negative *Staphylococcus*; *S. pneumoniae*/*S. mitis* group, no differentiation between *S. pneumoniae* and *S. mitis*; oral *Streptococcus* group, includes all oral species of streptococci except the *S. milleri* group; KES group, no identification between the species *K. pneumoniae* and *Enterobacter aerogenes* (KES, *Klebsiella-Enterobacter-Serratia*).

identification using conventional microbiological techniques. It should be pointed out that a precise identification among the group of oral streptococci is difficult to achieve with MALDI-TOF–MS; a Slidex pneumo-kit test (bioMérieux) was therefore performed on blood culture supernatant of the centrifuged positive blood culture fluid for either blood culture flasks spiked with *Streptococcus mitis* or *Streptococcus pneumoniae* or positive blood cultures identified as *S. pneumoniae* or *S. mitis* by MALDI-TOF–MS.

MALDI-TOF–MS. Two hundred microliters of the positive blood culture broth (or 1 ml of enrichment liquids) was transferred into a plastic tube containing 40 μl (or 200 μl for enrichment liquids) of a solution of 5% saponin to release intracellular bacteria. After 5 min of incubation at room temperature, distilled water was added up to 1.5 ml and 2 consecutive washes in distilled water were performed at 16,600 × *g* for 1 min. The supernatant was discarded, and 5 μl (or 30 μl for enrichment liquids) of 10% trifluoroacetic acid was added to the pellet. One microliter of this mixture was spotted (2 wells/sample) onto a MALDI sample target (Bruker Daltonics, Bremen, Germany) and allowed to dry at room temperature. One microliter of absolute ethanol was then added to each well, and the mixture was allowed to dry. One μl of DHB matrix solution (80 mg/ml 2,5-dihydroxybenzoic acid, 30% acetonitrile, 0.1% trifluoroacetic acid) was then added and allowed to cocrystallize with the sample. Samples were processed in a MALDI-TOF–MS spectrometer (Microflex; Bruker Daltonics) with Flex Control software (Bruker Daltonics). Positive ions were extracted with an accelerating voltage of 20 kV in linear mode. Each spectrum was the sum of the ions obtained from 400 laser shots performed automatically on different regions of the same well. The spectra were analyzed in an *m*/*z* range of 3,640 to 20,000 and compared with those of a reference database (Andromas, Paris, France). This database has been engineered as previously described and encompasses the pathogens encountered in human pathology (3, 5). The identification of the tested strain corresponds to the species of the reference strain having the best match in the database. The analysis also takes into account the difference between the first two species having the best matches with the reference database. The species identification was considered to be valid if, for one of the two sample deposits, the percentage of matched peaks was at least 60% of that of the first species proposed in the database after analysis by the Andromas software and if the difference between the first two species having the best match in the database is at least 10%. If the latter condition was not fulfilled, the identification

TABLE 2. Direct bacterial identification by MALDI-TOF–MS in monobacterial blood cultures from patients

Identification obtained from subculture (biochemical or molecular techniques)	No. of samples:					
	Tested	With identification by MALDI-TOF–MS to:				With unacceptable MALDI-TOF–MS profiles
		Species	Group[a]	Genus	Family	
Staphylococcus species						
S. epidermidis	121	118	3 (CNS group)			
S. haemolyticus	3	3				
S. hominis	20	20				
S. pasteuri	1	1				
S. capitis	3	2	1 (CNS group)			
S. aureus	43	42				1
S. lugdunensis	1	1				
Micrococcus luteus	1	1				
Streptococcus species						
S. pyogenes	8	5	3 (*S. pyogenes*/*S. dysgalactiae* group)			
S. mitis	8		8 (*S. pneumoniae*/*S. mitis* group)			
S. pneumoniae	1		1 (*S. pneumoniae*/*S. mitis* group)			
S. gordonii	1		1 (oral *Streptococcus* group)			
S. pasteurianus	1					1
S. salivarius	1		1 (oral *Streptococcus* group)			
S. oralis	2	2				
Enterococcus faecalis	6	3		2	1	
Enterococcus faecium	2	1			1	
Gram-negative nonfermenting bacilli						
Pseudomonas aeruginosa	20	17				3
Stenotrophomonas maltophilia	4	4				
Achromobacter xylosoxydans	2	2				
Burkholderia cenocepacia	1		1 (*B. cepacia*/*B. cenocepacia* group)			
Pseudomonas oryzihabitans	1	1				
Enterobacteriaceae						
Escherichia coli	41	40				1
Enterobacter cloacae	24	24				
Citrobacter freundii	9	6			1	2
Enterobacter aerogenes	7	5	1 (KES group)		1	
Klebsiella oxytoca	6	6				
Klebsiella pneumoniae	10	10				
Proteus mirabilis	8	8				
Serratia marcescens	2	2				
Salmonella enterica	3	3				
Haemophilus influenzae	1	1				
Candida albicans	11	11				
Total	373	339	20	2	4	8

[a] CNS group, coagulase-negative *Staphylococcus*; *S. pyogenes*/*S. dysgalactiae* group, no differentiation between *S. pyogenes* and *Streptococcus dysgalactiae*; *S. pneumoniae*/*S. mitis* group, no differentiation between *S. pneumoniae* and *S. mitis*; oral *Streptococcus* group, includes all oral species of streptococci except the *S. milleri* group; *B. cepacia*/*B. cenocepacia*, no differentiation between *B. cepacia* and *B. cenocepacia*; KES group, no differentiation between the species *K. pneumoniae* and *E. aerogenes* (KES, *Klebsiella-Enterobacter-Serratia*).

was considered to be correct at the level of the group/genus/family if the first two matches belonged to the same group/genus/family of bacteria. In all other cases, the results were considered irrelevant. It should be pointed out that most unreliable identifications were due to poor quality spectra. When the blood cultures contained several bacterial species as seen by Gram staining, databases specific for Gram-negative bacilli and/or Gram-positive cocci were used.

RESULTS

Identification of germs spiked into blood culture flasks. In order to determine whether an accurate identification of pathogens could be obtained from bacteria grown in liquid medium, pilot experiments were first performed using blood culture bottles spiked with commonly isolated pathogens. Figure 1 shows an example of a spectrum obtained with *Escherichia coli* grown in a blood culture bottle compared to the spectrum of the same strain obtained from an isolated colony. A total of 292 bacterial strains and 20 *Candida* species were spiked into blood culture bottles. The results are shown in Table 1. Of the 307 interpretable spectra (98%), MALDI-TOF–MS allowed a good identification at the species, group,

TABLE 3. Direct identification by MALDI-TOF–MS in blood cultures containing ≥2 germs

Bacterial type(s) found by Gram stain	Microorganism(s) isolated	No. of cultures	Identification[a] using:		
			General database	Gram-negative bacillus database	Gram-positive coccus database
Gram-positive cocci and Gram-negative bacilli	*Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus epidermidis*	1	*E. coli*	*E. coli*	*Enterococcus faecalis*
	Acinetobacter baumannii, *S. mitis*	2	*A. baumannii*	*A. baumannii*	*Streptococcus pneumoniae*/*Streptococcus mitis* group
	E. faecalis, *E. coli*	1	*E. faecalis*	0	*E. faecalis*
	Staphylococcus aureus, *Proteus mirabilis*	4	*S. aureus* (4/4)	*P. mirabilis* (2/4)	*S. aureus* (4/4)
	Pseudomonas aeruginosa, *S. epidermidis*	1	*P. aeruginosa*/*S. epidermidis*	*P. aeruginosa*	*S. epidermidis*
Gram-negative bacilli	*E. coli*, *Morganella morganii*	1	*E. coli*	*E. coli*	NA
	E. coli, *Klebsiella pneumoniae*	1	*E. coli*	*E. coli*	NA
	E. coli, *Proteus mirabilis*	1	*E. coli*	*E. coli*	NA
	K. pneumoniae, *E. cloacae*	1	KES	KES	NA
Gram-positive cocci	*E. faecalis*, *S. aureus*	1	*E. faecalis*	NA	*E. faecalis*
	Staphylococcus haemolyticus, *Staphylococcus hominis*	1	*S. hominis*	NA	*S. hominis*

[a] KES, *Klebsiella-Enterobacter-Serratia* group; NA, not applicable.

genus, and family level in 89%, 6%, 0.4% and 2.6% of cases, respectively. It should be pointed out that MALDI-TOF–MS allowed the differentiation of coagulase-negative staphylococci (CNS) from *Staphylococcus aureus* in 100% of cases. As already mentioned, precise identification among the group of oral streptococci by MALDI-TOF–MS remained difficult, and bacteria belonging to this group were subjected to a Slidex pneumo-kit test. Among the 12 *S. pneumoniae*/*S. mitis* strains spiked into blood culture bottles, only the *S. pneumoniae* strains were positive with the Slidex pneumo-kit test.

Identification of microbes in positive blood cultures from patients. Among the 388 positive blood cultures included in this study, 373 were monomicrobial (Table 2). Using MALDI-TOF–MS as described in Materials and Methods or a Slidex pneumo-kit test when the MALDI-TOF–MS identification was consistent with either *S. pneumoniae* or *S. mitis*, an interpretable identification was obtained in 98% of cases. These results were concordant with those obtained by classical methods at the species, group, and genus/family levels in 91%, 5%, and 2% of cases, respectively.

In addition, 15 patient blood cultures containing mixed bacteria were tested (Table 3). Using the database, either only one of the pathogens present in the mixture was detected or two pathogens were detected at the same score. When Gram-pos-

TABLE 4. Direct bacterial identification by MALDI-TOF–MS in enrichment cultures from patients

Source(s) of fluid sample(s) and identification obtained from subculture (biochemical or molecular techniques)	No. of samples:			
	Tested	Identified by MALDI-TOF–MS to:		
		Species	Group[a]	Genus
Graft conservation liquid				
E. coli	3	2	1 (*Shigella*/*E. coli*)	
Hafnia alvei	2	2		
Staphylococcus epidermidis	7	7		
Staphylococcus warneri	2	2		
Staphylococcus cohnii	1	1		
Articular fluid, bone puncture, deep abscess				
Staphylococcus aureus	16	16		
Staphylococcus epidermidis	5	5		
Staphylococcus capitis	1	1		
Staphylococcus lugdunensis	1	1		
E. coli	4	4		
Enterobacter cloacae	1	0		1
Pseudomonas aeruginosa	2	2		
Streptococcus pyogenes	1	1		
Total	46	44	1	1

[a] *Shigella*/*E. coli*, no differentiation between *Shigella* sp. and *E. coli.*

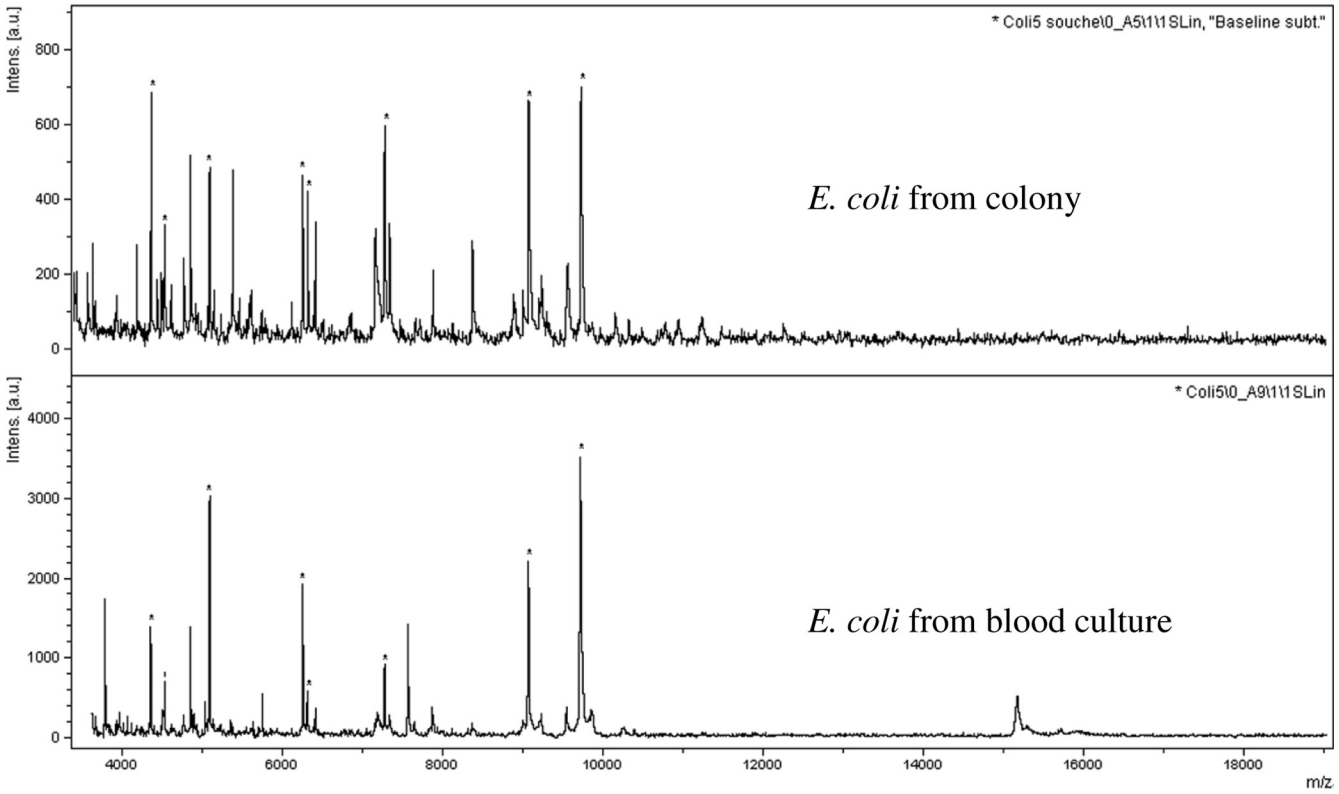

FIG. 1. Spectral profiles of the same strain of *Escherichia coli* obtained from blood culture (bottom) and from one isolated colony (top). Peaks in common between the two spectra are marked by asterisks. These peaks are specific to *E. coli*. Intens., intensity; a.u., arbitrary unit (absorption).

itive cocci and Gram-negative bacilli were detected by Gram staining, the identification was improved in 6 out of 9 cases by using a database containing species-specific spectra of Gram-positive cocci or Gram-negative bacilli.

Identification of microbes in positive enrichment fluids from blood culture flasks. We included 46 fluids grown in blood culture broths (Table 4). All spectra were interpretable, and we obtained an identification concordant with that obtained by classical methods at the species, group, and genus levels in 96%, 2%, and 2% of cases, respectively.

Time to diagnosis. It should be pointed out that each patient with a positive blood culture was treated as soon as it was detected as positive. The time required between the BacT/Alert alarm and the germ identification, including Gram staining performed during the incubation with detergent, was 20 min.

DISCUSSION

We evaluated the sensitivity and accuracy of pathogen detection by MALDI-TOF–MS applied directly from BacT/Alert bottles. This study enables a rapid (20 min) and reliable identification of the vast majority of microorganisms isolated in blood or fluid cultures. A rapid and accurate diagnosis diminishes the use of inadequate and broad-spectrum antibiotics, thereby improving outcome and reducing the potential development of resistance and possible side effects (1, 6, 10, 11). Identification of microorganisms in blood cultures by MALDI-TOF–MS dramatically extends the influence of the results of Gram staining on clinical management. In particular, among the Gram-negative bacilli, the differentiation of *Enterobacteriaceae* from members of the *Pseudomonas* or *Acinetobacter* genera only 20 min after the blood culture growth will allow a more appropriate treatment pending the results of susceptibility testing. Similarly, the possibility of obtaining an immediate diagnosis of *S. aureus* is of major clinical consequence. Fast differentiation of *S. aureus* from CNS should help the clinician to discriminate a serious infection from a possible contamination. The spectral profiles of *S. mitis* and *S. pneumoniae* are frequently indistinguishable. Nevertheless, we have shown with the results presented here and for 40 additional strains (20 *S. pneumoniae* and 20 *S. mitis* strains; data not shown) that the combination of a MALDI-TOF–MS identification result at the *S. mitis*/*S. pneumoniae* group level and a positive agglutination result with the Slidex pneumo-kit test allowed the two species to be discriminated with 95% specificity and 100% sensitivity (one test was uninterpretable because of an agglutination with the negative control). This differentiation has an important impact on the clinical management of patients.

Despite the good identification results, we noticed that the spectra from blood cultures were often of lower quality than those from colonies, occasionally making it difficult to differentiate among closely related species. For example, differentiation between *Burkholderia cepacia* and *Burkholderia cenocepacia* was not possible because of the lower quality of the spectra compared to those obtained from the colonies the next day. When the infection was due to several bacterial species,

the most abundant germ detected by Gram staining was in most cases identified by MALDI-TOF–MS. The identification of bacteria distinguishable by Gram staining required the use of specific Gram stain-based databases. However, a better algorithm may be needed to differentiate all mixtures of germs. In our hospital, in 2009, 2,555 blood cultures were found to be positive, and among these, 4.8% were polymicrobial (90% with two germs and 10% with three germs). Only 2.3% of polymicrobial blood cultures were not identified as such by Gram staining. MALDI-TOF identification of germs grown directly in blood culture flasks will therefore be a valuable tool to help the clinician to institute the initial antibiotic treatment.

Several studies have described different techniques designed to shorten the delay of bacterial identification in blood culture bottles, but none of these reach the level of performance of MALDI-TOF–MS. Indeed, according to de Cueto et al., only 62% of Gram-negative bacilli and 0% of Gram-positive cocci were properly identified by using direct inoculation of fluid from a positive blood culture into an automated identification system (4). Using similar systems, Kerremans et al. showed that same-day identification results were available for only 55% of patients (15). In addition, automatic rapid systems require 3.5 h for bacterial identification, versus only 20 min for MALDI-TOF–MS. PCR-based techniques have been used for bacterial identification directly from blood culture broth. Some methods require the use of specific targets (8, 19, 20, 24). Despite the fact that these techniques are sensitive, they remain expensive and are specific for one or a few pathogens. Many molecular approaches directed against several targets or one universal target have been successfully used to identify bacteria directly from positive blood culture bottles, but these methods are expensive and time consuming (22, 28, 29). Pyrosequencing is promising in its ability to differentiate multiple organisms in a positive blood culture, but this strategy is still restricted to research laboratories (12, 13). The use of fluorescent *in situ* hybridization (FISH) with oligonucleotides or peptide nucleic acid probes applied to growth-positive blood cultures is less labor intensive than PCR (21, 23, 26). Although the sensitivity and specificity of individual probes are good, identification at the species level is accurate in less than 80% of cases in routine use. Indeed, the usefulness of FISH as a diagnostic test depends on the probes included in the assay and is related to the epidemiology of microorganisms in a specific setting. In routine practice, FISH requires more than 4 h after Gram staining.

In summary, MALDI-TOF–MS is the fastest of all techniques for bacterial identification directly from blood culture broth, thus allowing a real-time diagnosis of bacteremia.

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